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The AT-hook DNA binding ability of the Epstein Barr virus EBNA1 protein is necessary for the maintenance of viral genomes in latently infected cells

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ABSTRACT

Epstein Barr Virus (EBV) is a human tumor virus that is causally linked to malignancies such as Burkitt's lymphoma, and gastric and nasopharyngeal carcinomas. Tethering of EBV genomes to cellular chromosomes is required for the synthesis and persistence of viral plasmids in tumor cells. However, it is not established how EBV genomes are tethered to cellular chromosomes. We test the hypothesis that the viral protein EBNA1 tethers EBV genomes to chromosomes specifically through its N-terminal AT-hook DNA-binding domains by using a small molecule, netropsin, that has been shown to inhibit the AT-hook DNA-binding of EBNA1 *in vitro*. We show that netropsin forces the loss of EBV genomes from epithelial and lymphoid cells in an AT-hook dependent manner and that EBV-positive lymphoma cells are significantly more inhibited in their growth by netropsin than are corresponding EBV-negative cells.

Introduction

Epstein Barr Virus (EBV) is a ubiquitous human pathogen associated with infectious mononucleosis and causally linked to several human malignancies (for a review see Sugden (2014)). There are close to 200,000 new cases of EBV-associated cancers worldwide every year, including Burkitt's lymphoma, which is among the most common childhood cancers in sub-Saharan Africa (Cohen et al., 2011).

In EBV-derived tumor cells the virus exists in a latent state of infection. Viral genomes are maintained as circular, extrachromosomal plasmids, which are replicated in a licensed manner during the S-phase of the cell cycle and subsequently segregated to daughter cells equally and non-randomly during mitosis (Adams, 1987; Yates and Guan, 1991; Nanbo et al., 2007). In addition to cellular factors, only two viral elements are necessary for the maintenance of EBV plasmids in latently infected cells: a portion of the EBV genome called *oriP* and the protein EBNA1 (Yates et al., 1984, 1985; Lupton and Levine, 1985). OriP consists of two sets of repeated EBNA1 binding sites, DS (Dyad Symmetry), which functions as an EBNA1-dependent replication origin (Gahn and Schildkraut, 1989; Schepers et al., 2001) and FR (Family of Repeats), which has been shown to be necessary for the persistence of EBV and EBV-derived plasmids in latently infected cells (Kanda et al., 2001).

EBNA1 is a homo-dimeric, multi-functional DNA-binding protein. It can site-specifically bind to sequences in EBV genomes (Ambinder et al., 1990; Frappier and O'Donnell, 1991) and cellular chromosomes (Dresang et al., 2009; Canaan et al., 2009; Lu et al., 2010) through a C-terminal DNA-Binding and Dimerization domain (DBD). EBNA1 also contains two N-terminal domains called Linking Region 1 (LR1; aa 33–89) and Linking Region 2 (LR2; aa 325–376), which have been shown to be able to bind mitotic chromosomes in cells and AT-rich DNA and G-quadruplex RNA structures *in vitro* (Marechal et al., 1999; Sears et al., 2004a; Norseen et al., 2009).

The replication and partitioning of EBV genomes and EBV-derived plasmids requires them to be tethered to cellular chromosomes (Kanda et al., 2001; Holdin et al., 2013). Evidence indicates that EBNA1 facilitates the association of EBV genomes to cellular chromosomes by binding multiple sites in the FR portion of oriP through its C-terminal DBD and tethering the viral plasmids to cellular DNA through N-terminal domains (for a review see Frappier (2012) and Lindner and Sugden (2007)). Although it remains unclear exactly how LR1 and LR2 interact with cellular DNA when EBNA1 is bound to FR through the C-terminal DBDs at least three non-exclusive mechanisms have been suggested.

1) The cellular protein EBP2 has been proposed to mediate EBNA1's association with chromosomes to facilitate the tethering of EBV

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genomes to cellular DNA (Shire et al., 1999; Kapoor et al., 2005). A version of EBNA1 with LR2 deleted, ΔLR2-EBNA1, did not bind EBP2 and cells expressing ΔLR2-EBNA1 could not maintain EBV-derived plasmids as efficiently as cells expressing wtEBNA1 (Shire et al., 1999). Also, depletion of EBP2 from cells led to a shift of EBV-derived plasmids from the chromosomal to the soluble fraction of cell lysates (Kapoor et al., 2005). However, other research has shown the presence of LR1 alone is enough to localize EBNA1 to mitotic chromosomes, and the failure of ΔLR2-EBNA1to support EBV-derived plasmid maintenance could be complemented by expressing versions of ΔLR2-EBNA1 containing multiple repeats of LR1 (Sears et al., 2004a). In addition, while EBP2 and EBNA1 appeared to colocalize in interphase cells, there are contradictory findings as to whether and when they do so during mitosis (Nayyar et al., 2009; Jourdan et al., 2012). Ultimately it remains unclear what role EBP2 plays in the tethering of EBV genomes to cellular chromosomes and the maintenance of viral DNAs in latently infected cells, although recent hypotheses postulate that EBP2 stabilizes EBNA1-chromatin interactions during mitosis (Frappier, 2012).

2) LR1 and LR2 have been shown to bind G-rich RNA that is predicted to form G-quadruplex structures (Norseen et al., 2009). A G-quadruplex-interacting compound inhibited the association of EBNA1 with mitotic chromosomes and EBNA1-dependent replication at oriP. In addition, culturing EBV-positive cells in the presence of a G-quadruplex interacting compound reduced the genome copy number and inhibited the growth of those cells (Norseen et al., 2009). However, the ability of LR1 and LR2 to interact with G-rich RNA has also shown to be important for the recruitment of the origin recognition complex (ORC) at the DS portion of OriP (Norseen et al., 2008). Thus it is unclear whether the reduction in EBV genome copy number in the presence of G-quadruplex-interacting compounds is due to inhibition of EBNA1's ability to bind chromosomes and/or a reduction in EBNA1-ORC interactions.
3) EBNA1's LR1 and LR2 contain AT-hook DNA-binding domains and are able to bind specifically to AT-rich DNA *in vitro* (Sears et al., 2004a). In addition, the entire N-terminal half of EBNA1 can be replaced by cellular proteins that contain AT-hook DNA-binding domains, such as HMGA1, and the fusion protein is able to mediate persistence of EBV-derived plasmids in cells (Sears et al., 2004b). However, other cellular and viral proteins that bind chromosomes, but lack AT-hook DNA-binding domains, such as the cellular protein Histone H1 or the first 22 amino acids of the LANA1 protein from Kaposi's Sarcoma-associated Herpes Virus (KSHV) are also able to complement the loss of EBNA1's LR1 and LR2 to maintain oriP replicons (Holdin et al., 2013; Hung et al., 2001).

Because LR1 and/or LR2 are involved in all the proposed mechanisms by which EBNA1 tethers EBV genomes to cellular DNA it has been challenging to test specifically whether EBNA1's ability to bind AT-rich DNA is necessary *in vivo* for it to be able to maintain EBV genomes in cells. We hypothesized that we could use small molecules that bind specifically to AT-rich DNA and have been shown to disrupt the DNA-binding of EBNA1 LR1 and LR2 *in vitro* (Sears et al., 2004a) to determine whether the AT-hook activity of EBNA1 is necessary *in vivo* for the maintenance of EBV genomes in latently infected cells. These small molecules are not expected to disrupt the ability of EBNA1 to bind EBP2 or G-quadruplex RNA since they interact with AT-rich DNA and not EBNA1 itself.

We used netropsin, which is a naturally-derived small molecule that binds to the minor groove of AT-rich DNA (reviewed by Bailly and Chaires, 1999). We reasoned that small molecules that bind AT-rich DNA and inhibit EBNA1's binding to AT-rich DNA *in vitro* would also inhibit its binding AT-rich DNA *in vivo*. If the AT-hook domains of EBNA1 are responsible for tethering viral genomes to chromosomes in latently infected cells, their inhibition by netropsin would lead to a loss of EBV genomes from these cells.

We have extended previous *in vitro* results to show that inhibition of EBNA1's AT-Hook functions by netropsin leads to a loss of EBV genomes from several different cell lines, including tumor cells, in an AT-hook dependent manner. In addition, culturing tumor cells dependent on EBV in the presence of netropsin inhibits their growth significantly more than that of EBV-negative tumor cells.

Our results highlight a vital and necessary role for the AT-hook binding ability of EBNA1's LR1 and LR2 in the maintenance of EBV genomes in latently infected cells, such as tumor cells.

Methods

Cell lines and culture conditions

The Wp-restricted BL cell lines OkuI (Kelly et al., 2002) and SalI (Kelly et al., 2002) expressing Bcl-XL and the PEL cell lines JSC-1 (Cannon et al., 2000) and BC-1 (Cesarman et al., 1995) were cultured in RPMI 1640 (Invitrogen) supplemented with L-glutamine, 10% fetal bovine serum (FBS), and antibiotics (200 U/mL penicillin and 200 μg/mL streptomycin). HEK293 (Graham et al., 1977) cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with L-glutamine, 10% FBS and antibiotics (D10F). HEK293 cells harboring p2089, p294-wtEBNA1, p4880-H1 or p4881-LANA1 were grown in D10F and 400 μg/mL hygromycin. HEK293 cells expressing EBNA1 were obtained from the ATCC (CRL-10852) and grown in D10F with 250 μg/mL G418. All cells were incubated in 5% CO2 at 37 °C.

Reagents

Netropsin dihydrochloride was purchased from either Sigma-Aldrich (N9653-5 MG) or Enzo Life Sciences (ALX-380-088-M005).

Recombinant DNA plasmids

p2089, the recombinant B95.8 EBV genome with eGFP and hygromycin resistance gene added, has been previously described (Delecluse et al., 1998). p294 has also been described previously as CMVpEBNA1 (Sugden and Warren, 1989). To generate plasmids encoding EBNA1-DBD fused to either Histone H1 or the first 22 amino acids of KSHV LANA1 we first generated p294-NoEBNA1 by digesting p294 with MfeI and XbaI (NEB). DNA oligonucleotides encoding the Histone H1 protein, the first 22 amino acids of KSHV LANA1 or the C-terminal domain (aa 376–641) of EBNA1 were ordered as Geneblocks (IDT DNA). These geneblocks were subcloned into the p294-NoEBNA1 using the GIBSON Assembly Cloning Kit (New England BioLabs) according to manufacturer's instruction. The final assemblies were verified by sequencing.

Transfections

293 cells were plated in 60-mm dishes and grown to ~80% confluence. For each dish, 10 μg of vector and 1ug of reporter plasmid was diluted in 250 μl of Opti-MEM (Invitrogen) and then combined with pre-diluted Lipofectamine-Opti-MEM (Invitrogen) mixture (8 μl of Lipofectamine diluted in 250 μl of Opti-MEM, incubated 5 min at room temperature). DNA-Lipofectamine complexes were incubated for 25 min at room temperature. Cells were washed once with PBS and plated with 1.5 mL DMEM. The 500 μl transfection mixture was added and the plates incubated for 4 h at 37 °C at 5% CO2. Following the incubation, the medium was replaced with 5 mL of fresh D10F. The transfection efficiency was determined ~48 h later by counting GFP-positive cells.

Growth curve analysis

Cells were plated at a density of between 4×10^5 and 2×10^5 cells/ml of culture medium. Live cell concentrations were measured at specified intervals with a haemocytometer. Cells stained with trypan blue (with a 1:10 dilution of 0.3% trypan blue dissolved in phosphate-buffered saline [PBS]) or exhibiting aberrant morphologies were considered nonviable. After counting, when necessary, cells were diluted in fresh medium back to their approximate starting concentration.

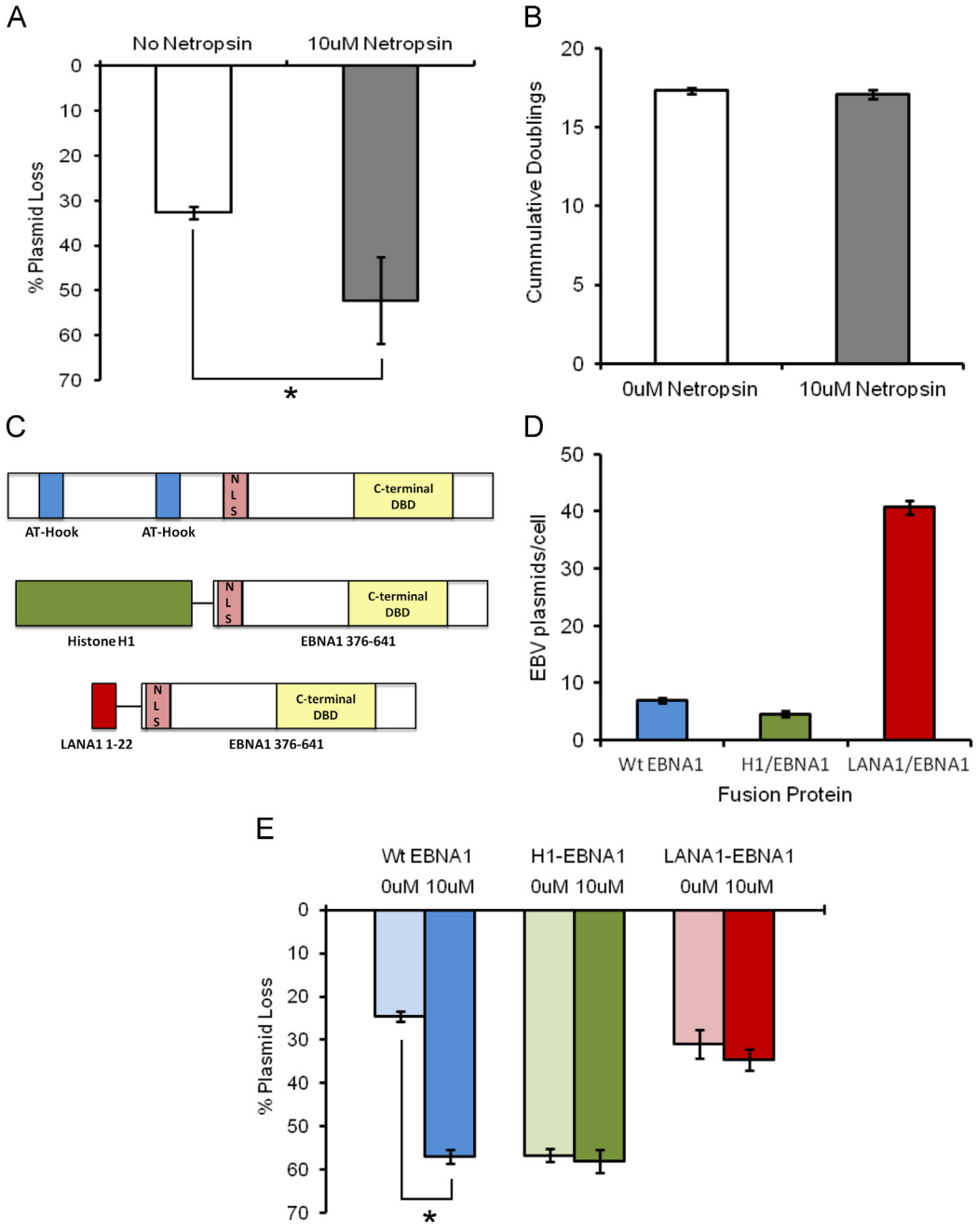

Fig. 1. Netropsin increases the rate of loss of EBV plasmids from 293 cells in an EBNA1 AT-hook dependent manner. (A) Netropsin increases the rate of loss of EBV plasmids from 293 cells. 293 cells harboring recombinant EBV plasmid (p2089) were cultured in the absence of Hygromycin and with either no netropsin (white bar) or 10 μM netropsin (gray bar) for 18 days. The number of EBV genomes/cell was determined using qRT-PCR and the reduction in the number of viral genomes per cell after 18 days ± netropsin is shown as % plasmid lost. (B) Culturing 293 cells with 10 μM netropsin does not affect their growth rate. 293 cells harboring 2089 were cultured as in (A). Live cells were counted using a Trypan Blue exclusion assay and the number of doublings over 18 days was calculated. (C) Fusion proteins were made with the C-terminal portion of EBNA1 (aa 376–641) and either the first 22 amino acids of the KSHV LANA1 protein or the cellular histone H1 protein. The EBNA1 C-terminal domain used retained the native Nuclear Localization Signal (NLS) and the DNA-Binding and dimerization Domain (DBD). The peptides were linked using a (GGGGSx2) linker. (D) EBV-derivative plasmids can be maintained in 293 cells expressing the C-terminal portion of EBNA1 fused to either histone H1 (green bar) or the first 22 amino acids of the KSHV LANA1 protein (maroon bar). "Wt EBNA1" refers to the parental p294-wtEBNA1 plasmid. (E) Netropsin increases the rate of loss of EBV-derived plasmids from 293 cells expressing wt-EBNA1 (blue bars) but not from cells expressing LANA1- or H1- fusions with the C-terminal domain of EBNA1. 293 cells expressing wt-EBNA1, H1-EBNA1 (green bars) or LANA1-EBNA1 (maroon bars) and harboring EBV-derived plasmids were grown with or without 10 μM netropsin for 18 days. The average number of EBV plasmids was determined at day 18 using qRT-PCR and the reduction in the number of viral genomes per cell after 18 days ± netropsin is shown as % plasmid lost. For all figures the average of at least three independent trials is presented and the error bars show standard deviations [$*=p<0.05$].

Fluorescence in situ hybridization

EBV-positive OkuI, SalI or JSC-1 Cells were prepared as described and hybridized with probes that were generated as described (Nanbo et al., 2007). At least 40 cells were counted per replicate for each FISH assay.

DNA extraction and quantitative RT-PCR (qPCR)

DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen). Real-time PCR was carried out as previously described (Vereide and Sugden, 2011).

Statistics

Statistical analyses were performed using either Microsoft Excel or MStat software, version 5.5 (N Drinkwater, McArdle Laboratory for Cancer Research, School of Medicine and Public Health, University of Wisconsin) and is available for downloading at: http://www.mcardle.wisc.edu/mstat. At least three biological replicates and the Student's *t*-test were used for statistical analyses unless otherwise stated.

Results

Netropsin increases the rate of loss of EBV plasmids from 293 cells in an EBNA1 AT-hook dependent manner

If netropsin inhibits the ability of EBNA1's AT-hooks to bind DNA and tether EBV genomes to cellular chromosomes in latently infected cells we would expect to see loss of the viral DNAs from those cells, as tethering has been shown to be necessary for the replication and partitioning of viral plasmids (Kanda et al., 2001; Holdin et al., 2013). But latently infected cells, including tumor cells, depend on EBV for growth and survival, and those cells that lose viral genomes die by apoptosis (Vereide and Sugden, 2011; Kennedy et al., 2003). Therefore, it would be difficult, if not impossible, to measure accurately loss of EBV genomes from these cells. To overcome this obstacle we developed an approach using cells that are conditionally dependent on EBV DNA for growth and survival.

We used 293 cells which harbor a recombinant version of EBV called p2089, which comprises the B95.8 EBV genome and expresses GFP and a gene encoding resistance to hygromycin (Delecluse et al., 1998). When 293_p2089 cells are cultured in the presence of hygromycin, p2089 persists in them, but in the absence of hygromycin these cells are not dependent on p2089 for survival or growth and the plasmids are lost over time as a result of defects in their synthesis (Nanbo et al., 2007). We cultured 293_p2089 cells either in the presence or absence of netropsin, but the absence of hygromycin, for 18 days (approximately 20 cell generations), to test whether netropsin would increase the rate of loss of p2089. We extracted DNA after 18 days and determined the average number of p2089 plasmids/cell using quantitative PCR (q-PCR).

Culturing 293_p2089 cells in the presence of 10 μM netropsin increases the rate of loss of p2089 plasmids (Fig. 1A) without having any inhibitory effect on the growth or viability of these cells (Fig. 1B, Supplementary Fig. 1A). We did not use concentrations of netropsin greater than 10 μM because at those higher concentrations the 293_2089 cells showed both slower growth and death as measured by Trypan Blue exclusion assays (data not shown).

To confirm that netropsin was specifically affecting the AT-hook activity of EBNA1 in the cells we tested, we replaced the N-terminal portion of EBNA1, which contains the AT-hook DNA-binding domains, with either the histone H1protein or the first 22 amino acids of the LANA1 protein from Kaposi's Sarcoma-associated Herpes Virus (KSHV). KSHV genomes appear to be tethered to cellular DNA through attachments to Histone H2A and H2B mediated by LANA1, a viral protein (Barbera et al., 2006, Shinohara et al., 2002). We generated plasmids encoding these fusion proteins (p4880-H1 and p4881-LANA1 (1–22) respectively), the *OriP* portion of the EBV genome and a gene encoding resistance to hygromycin (Fig. 1C). Both the H1/EBNA1-DBD and the LANA1:1–22/EBNA1-DBD fusion proteins lack any AT-hook DNA-binding domain, but we confirmed previous results showing that both are able to maintain EBV-derived plasmids in 293 cells in the presence of selection, as are 293 cells harboring the parental p294-wtEBNA1 (Fig. 1D) (Holdin et al., 2013; Hung et al., 2001).

We cultured 293 cells selected to harbor p294-wtEBNA1, p4880-H1 or p4881-LANA1 in the absence of hygromycin and in the presence or absence of 10 μM netropsin for 18 days. We hypothesized that netropsin would increase the rate of loss of p294-wtEBNA1 but not that of p4880-H1 or p4881-LANA1 from these 293 cells as, unlike wt EBNA1 with AT-hook binding domains, the fusion proteins are not expected specifically to interact with AT-rich DNA. Cells were collected after 18 days, DNA was extracted and the average number of p294-wtEBNA1, p4880-H1 or p4881-LANA1 per cell was determined using q-PCR. Unlike plasmids in 293 cells expressing wild-type EBNA, which showed increased rates of loss in the presence of netropsin, the rate of loss of p4880-H1 and p4881-LANA1 plasmids, although different from each other, did not increase when the cells maintaining these plasmids were cultured in the presence of 10 μM netropsin (Fig. 1E). In addition the loss of the EBV-derived plasmids in the absence of selection confirmed the extrachromosomal nature of these DNAs.

Netropsin forces the loss of EBV, but not KSHV, genomes from dually-infected PEL cells

Dually-infected primary effusion lymphomas (PELs) contain both EBV and KSHV genomes as plasmids (Nador et al., 1996). We tested whether culturing PELs in the presence of netropsin would induce the loss of either EBV or KSHV genomes from these tumor cells. Since KSHV genomes are not tethered to cellular DNA via AT-hooks we predicted that netropsin would force the loss of EBV but not KSHV genomes from dually-infected PEL cells.

We cultured JSC-1 PEL cells in the presence of 10 μM of netropsin for 18 days. At this concentration, netropsin did not have an adverse effect on the doubling time of these cells (Fig. 2A), and neither did we see an increase in the number of dead or dying cells using Trypan Blue exclusion assays (Supplementary Fig. 1 A). Cells cultured in netropsin were collected after 18 days and the numbers of EBV and KSHV genomes/cell were determined by FISH (EBV) and/or qPCR (EBV and KSHV). Because JSC-1 cells contain more than 100 copies of KSHV genomes per cell, we used qPCR to assay the number of KSHV genomes. We found no significant difference between using FISH and qPCR to follow the loss of viral genomes in the presence of netropsin (Supplementary Fig. 1B and 1C).

Netropsin caused a significant loss of EBV plasmids in JSC-1 cells but did not decrease the number of KSHV genomes/cell in JSC-1 cells (Fig. 2B), showing that its effect is specific to EBV and EBNA1. These results were replicated using dually-infected BC-1 PEL cells (Fig. 2C and D).

Netropsin inhibits the growth of EBV-positive Wp-restricted BL cells significantly more than that of EBV-negative BL cells

Having established that netropsin can increase the rate of or induce the loss of EBV genomes from different cell lines, we next tested whether culturing tumor cell lines dependent on EBV for survival in the presence of netropsin would A) lead to a decrease in the average number of EBV genomes/cell and, as would be

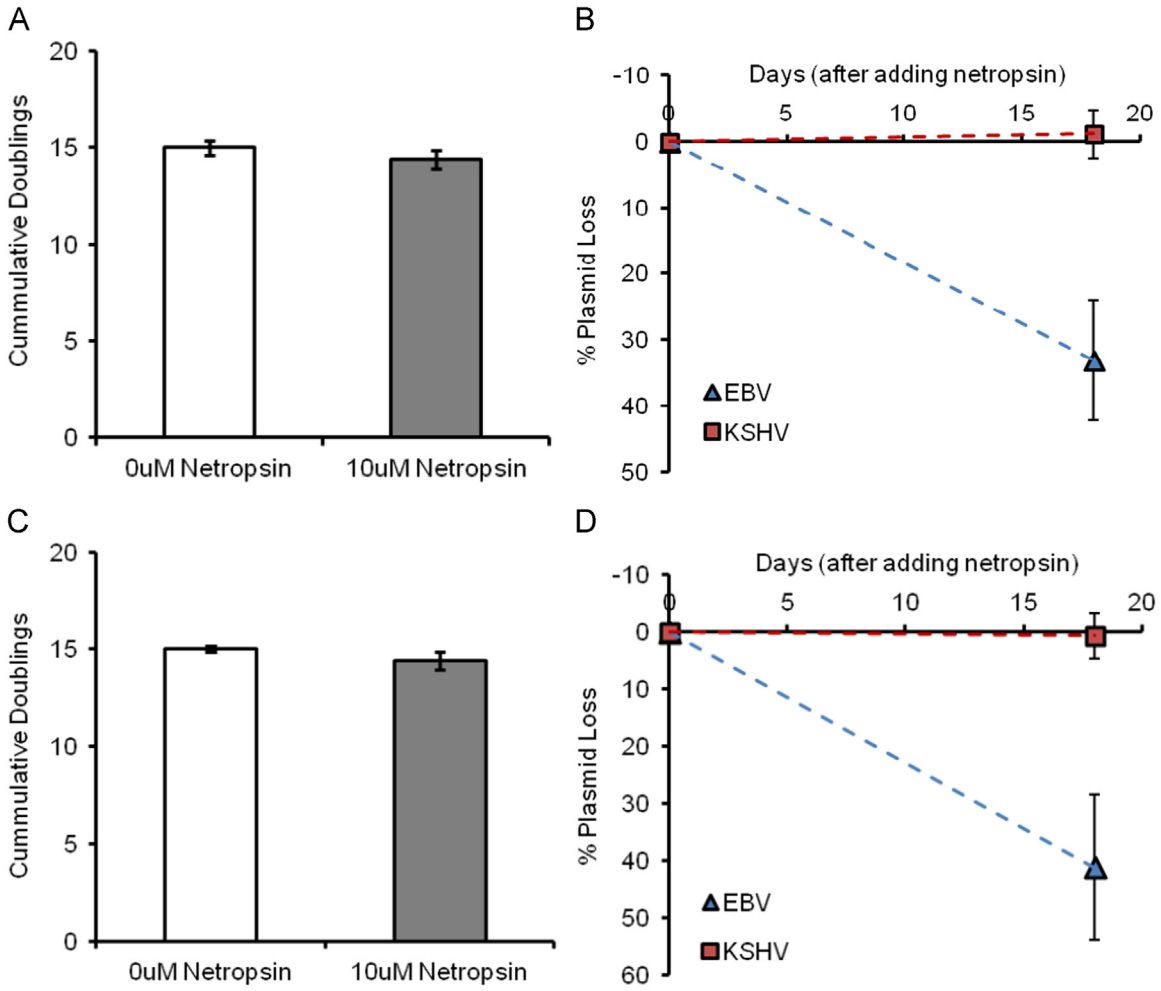

Fig. 2. Netropsin forces the loss of EBV but not KSHV genomes from dually infected Primary Effusion Lymphoma cells. (A) Culturing JSC-1 cells with 10 μM netropsin does not affect their growth rate. JSC-1 cells were cultured in the absence of netropsin (white bar) or 10 μM netropsin (gray bar) for 18 days. Live cells were counted using a Trypan Blue exclusion assay and the number of doublings over 18 days was calculated. (B) Netropsin forces the loss of EBV, but not KSHV, genomes from JSC-1 cells. Dually-positive JSC-1 cells were cultured as in (A). The number of EBV (blue triangles) and KSHV (red squares) genomes per cell was determined using FISH or qRT-PCR. The reduction in the number of viral genomes in cells cultured in the presence of netropsin compared to number of genomes in cells cultured without netropsin was calculated as % plasmid lost. At least 80 cells were counted per replicate. (C), (D) These results were replicated for BC-1 cells. For all figures the average of at least three independent experiments is shown. Error bars show standard deviations.

expected, B) inhibit their growth concomitant with this loss of viral genomes.

We have shown that forcing the loss of EBV genomes from Wp-restricted BL cells causes them to die by apoptosis (Vereide and Sugden, 2011). When we cultured OkuI BL cells in the presence of various amounts of netropsin we observed, as expected, a dose-dependent decrease in the number of live cells over time (Supplementary Fig. 2). We have also shown that exogenous expression of the cellular anti-apoptotic protein Bcl-XL allows Wp-restricted BL cells that have lost EBV to survive and continue to proliferate (Vereide and Sugden, 2011). Therefore, we followed the loss of viral genomes from Wp-restricted BL cells expressing Bcl-XL to avoid the ambiguities resulting from analyzing dead and dying cells. We cultured EBV-positive SalI-BL cells that exogenously express Bcl-XL in the presence of 50 μM netropsin for 15 days. We used 50 μM netropsin as this is the lowest concentration of netropsin tested at which there is a continuous inhibition of growth of parental Wp-restricted BL cells (Supplementary Fig. 2A). Cells were counted every three days and live/dead cells were differentiated using Trypan Blue staining. After 15 days, cells were collected and the number of EBV genomes/cell was determined using FISH.

SalI+Bcl-XL cells cultured with netropsin for 15 days had a significant decrease in the number of genomes per cell compared to SalI+Bcl-XL cells cultured in the absence of netropsin (Fig. 3A). There was also a shift in the distribution of plasmids/cell and a significant increase in the percentage of cells with no EBV signal (Fig. 3B). These experiments were replicated with OkuI+Bcl-XL BL cells (Fig. 3C and 3D).

Previous research (Vereide and Sugden, 2011), (Vereide et al., 2014) has shown that while expression of Bcl-XL protects cells from apoptosis upon loss of EBV genomes, the rate of proliferation of these newly EBV-negative cells is decreased significantly. Therefore culturing EBV-positive SalI+Bcl-XL cells in the presence of netropsin should inhibit their growth concomitant with the observed loss of viral genomes. Conversely, EBV-negative SalI+Bcl-XL cells, which already have a slower growth rate due to loss of EBV genomes should not be affected by the presence of netropsin.

Netropsin significantly inhibited the growth of EBV-positive SalI+Bcl-XL cells. The growth of EBV-negative SalI+Bcl-XL cells was also inhibited by netropsin but to a significantly lesser extent than that of the EBV-positive SalI+Bcl-XL cells (Fig. 3E). We conclude, therefore, that at least part of the decrease in the growth

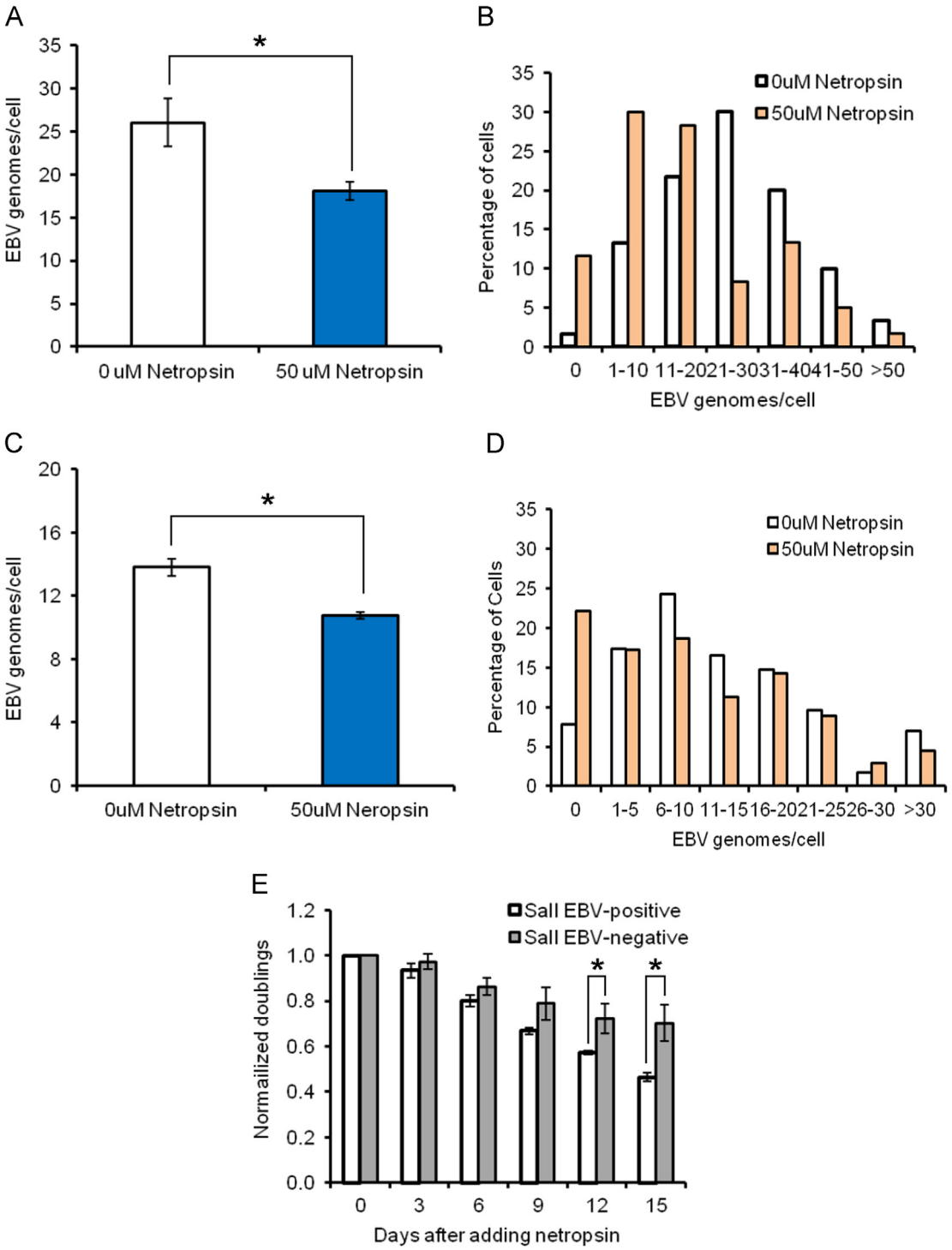

Fig. 3. Netropsin forces the loss of EBV genomes from Wp-restricted Burkitt's Lymphoma cells and inhibits their growth. (A) Netropsin forces the loss of EBV plasmids from SalI BL cells. EBV-positive SalI cells were cultured with either no netropsin (white bar) or 50uM netropsin (blue bar) for 15 days. The number of EBV genomes per cell was determined using FISH. The average of three independent experiments is presented with error bars showing standard deviations [$^{*}=p<0.05$]. (B) Culturing SalI BL cells with netropsin changes the distribution of EBV genomes/cell and increases the percentage of cells with no EBV genomes. SalI cells were cultured with and without netropsin as in (A) and the distribution of EBV genomes in cells $\pm$ netropsin was determined using FISH. The distribution of cells with different number of EBV genomes is presented as a percentage of the total number of cells counted ($n=120$). (C), (D) These results were replicated with OkuI-BL cells with experiments over 7 days. (E) Netropsin inhibits the growth of EBV-positive SalI BL cells significantly more than that of EBV-negative SalI BL cells. EBV-positive (white bar) and EBV-negative (blue bar) SalI + Bcl-XL BL cells were grown as in (A). Live cells were counted every 3 days using a haemocytometer and Trypan Blue exclusion assay. Cumulative doublings were calculated for EBV $\pm$ SalI BL cells grown $\pm$ netropsin. The number of doublings at each time point for SalI BL cells grown in the presence of netropsin was normalized to the number of doublings by cells grown in the absence of netropsin. In the absence of netropsin doublings times did not change over the duration of the experiment. The average of three independent experiments is presented. Error bars show standard deviations [$^{*}=p<0.05$].

rate of EBV-positive SalI+Bcl-XL cells in the presence of netropsin results from the induced loss of EBV genomes.

Discussion

EBV DNA persists in latently infected cells, including tumor cells, as extrachromosomal plasmids. The replication of these plasmids during the S-phase of the cell cycle and their subsequent partitioning to daughter cells during mitosis and cell division requires them being tethered to cellular chromosomes (Kanda et al., 2001; Holdin et al., 2013; Sears et al., 2004b)].

Two domains in the N-terminal portion of the EBV protein EBNA1—LR1 and LR2—have been shown to play a crucial role in mediating the association of viral genomes to cellular DNA (Sears et al., 2004a, 2004b; Norseen et al., 2009; Wu et al., 2002). But the mechanism by which these two domains mediate tethering of EBV genomes to cellular chromosomes has been unclear because LR1 and LR2 have been shown to have multiple functions including interacting with DNA, RNA, and cellular proteins.

We reasoned that we could use the small molecule netropsin, which binds to the minor groove of AT-rich DNA and inhibits LR1 and LR2 binding to poly-dA.dT DNA *in vitro* (Sears et al., 2004b), to inhibit specifically the AT-hook binding of EBNA1's LR1 and LR2 to AT-rich DNA *in vivo* without impinging on its ability to interact with G-rich RNAs (Norseen et al., 2009) or other cellular proteins (Shire et al., 1999; Jourdan et al., 2012).

By culturing cells that maintain recombinant EBV genomes with netropsin we showed that specifically inhibiting the AT-hook binding ability of EBNA1 increases the rate of loss of the recombinant EBV genomes from these cells. This increased loss of viral genomes occurred without affecting the growth of epithelial (293) and lymphoid (JSC-1 and BC-1) cells showing that this loss of EBV DNA is unrelated to the cytotoxic effects seen with netropsin when used at higher concentrations.

Netropsin does not increase the rate of loss of EBV derivatives when the AT-hook domains of EBNA1 are replaced by H1 or the N-terminal 22 amino acids of KSHV LANA1 or force the loss of KSHV genomes from dually-infected PEL cells. The induced loss of EBV replicons by netropsin depends on EBNA1's AT-hooks, which confirms the hypothesis that the AT-hook DNA-binding ability of EBNA1 LR1 and LR2 is necessary for the maintenance of EBV genomes in latently infected cells.

Interestingly, we and others have shown that the entire N-terminal half of EBNA1 can be replaced by proteins with or without AT-hook DNA-binding domains that maintain EBV-derived plasmids (Holdin et al., 2013; Sears et al., 2004b; Hung et al., 2001).These seemingly contradictory findings together indicate an EBNA1-fusion protein must be able to bind mitotic chromosomes by some means in order to maintain EBV-derived plasmids in cells.

Culturing Wp-restricted BL cells in the presence of netropsin caused a significant decrease in the average number of EBV genomes per cell and increased the proportion of cells with no EBV genomes. This loss of EBV genomes in the presence of netropsin significantly inhibited the growth of these EBV-positive BL cell lines expressing Bcl-XL. While netropsin also inhibited the growth of EBV-negative Wp-restricted BL cells, likely due to interfering with ORC binding to AT-rich cellular sequences (Vashee et al., 2003), we found that netropsin inhibits the growth of EBV-positive BL cells significantly more than that of the EBV-negative cells.

EBV is linked to several human malignancies and inhibiting functions of the viral protein EBNA1 is a compelling potential treatment for these cancers (Sun et al., 2010; Kang et al., 2012; Thompson et al., 2010; Jiang et al., 2014). Small molecules that inhibit the binding of EBNA1's AT-hooks to cellular DNA, and thereby force its loss from cells, have potential as therapies against EBV-related malignancies because EBV has been shown to be required to maintain these tumors (Vereide and Sugden 2011; Kennedy et al., 2003). Netropsin is too toxic to use in patients, but our results indicate molecules that directly bind the AT-hook domains of EBNA1 should force the loss of EBV genomes from tumor cells and be therapeutically beneficial.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.05.018.

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